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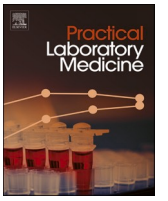

Clinical performance of a quantitative pan-genus *Leishmania* Real-time PCR assay for diagnosis of cutaneous and visceral leishmaniasis

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ABSTRACT

Leishmaniasis is a complex vector-borne disease caused by various *Leishmania* species, affecting humans and animals. Current diagnostic methods have limitations, leading to potential misdiagnosis. Therefore, there is an urgent need for specific and sensitive diagnostic tools. We evaluated the sensitivity of a quantitative real-time PCR (qPCR) assay targeting the 18S gene in diverse clinical sample matrices. The assay showed a wide dynamic range and a limit of detection (LoD) of 1 parasite equivalent per milliliter (eq-p/mL) for all tested species. It exhibited high specificity for *Leishmania* DNA, with no amplification against other microorganisms. When applied to samples from patients with visceral and cutaneous leishmaniasis, the qPCR assay provided results that matched the reference methods and allowed estimation of parasite burdens. This assay holds promise for diagnosing and monitoring leishmaniasis by offering high sensitivity, specificity, and the ability to estimate parasitemia. Further studies are needed to enhance *Leishmania* molecular diagnostics and expand their coverage for improved clinical impact.

Leishmaniasis encompasses a spectrum of diseases caused by flagellated protozoan of the genus *Leishmania*. The disease is caused by around 20 different species of *Leishmania*, each contributing to distinct clinical manifestations in humans and vertebrates. These manifestations include cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL), a severe chronic infection that can be fatal if left untreated [1,2].

Microscopic examination has traditionally served as the diagnostic method for CL and MCL, while serology has been regarded as the gold standard for VL. In recent years, polymerase chain reaction (PCR) techniques have emerged as more sensitive alternatives for the diagnosis and identification of *Leishmania* species. Various PCR platforms, including conventional PCR, real-time PCR, digital droplet

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PCR, and loop-mediated isothermal amplification (LAMP), have exhibited sensitivity ranging from 92 to 100 % and a specificity of 100 %. Nonetheless, the majority of these methods are qualitative in nature, with limited utilization of quantitative tools to estimate parasitic loads across different clinical phenotypes [3,4].

A recent study conducted by Filgueira et al. [5] evaluated the performance of a quantitative PCR (qPCR) assay utilizing two commonly targeted genetic markers, namely 18S rDNA and Heat Shock Protein 70 (HSP70), along with a commercially available RNAseP kit as an internal control. This assay demonstrated a sensitivity of 98.5 % and a specificity of 100 % when testing lesions from CL and MCL patients. However, the study presented certain limitations, including the establishment of a limit of detection (LoD) solely based on *L. braziliensis*, validation restricted to tegumentary leishmaniasis samples, and reliance on a commercial RNAseP kit for further validation. This latter aspect poses challenges, particularly in resource-constrained endemic regions. Therefore, this study aims to assess the analytical and diagnostic performance of the previously employed 18S rDNA qPCR assay, employing human RNAseP as an in-house internal control, using diverse clinical samples from patients with CL and VL, thereby circumventing the aforementioned limitations.

Experiments were conducted using three reference strains of *Leishmania*, namely MHOM/BR/75/M2903 *L. braziliensis*, MHOM/TN/80/IPT1 *L. infantum*, and IFLA/BR/67/PH8 *L. amazonensis*. DNA extraction was performed using the High Pure PCR Template Preparation kit, and serial dilutions were prepared from a stock containing 10^3 parasite equivalents/mL (These were quantified by Neubauer chamber). Human DNA from donors negative for Leishmaniasis and Chagas Disease was used as the dilution matrix.

A previously designed quantitative polymerase chain reaction (qPCR) assay targeting the 18S fragment was employed. The qPCR assay also included the human RNAseP gene as an endogenous control. The qPCR reactions were carried out using FastStart Universal Master Mix, specific primers and probes, and DNA templates as reported elsewhere [5]. The qPCR assays were performed using a Quantstudio 5 instrument, and the threshold was set at 0.02 for all targets.

The limit of detection (LoD) was determined as the lowest dilution that provided 95 % positive results using the calibration curves previously mentioned in triplicates. Probit regression analysis was used to calculate the LoD. The analytical specificity of the assay was evaluated by testing 44 DNA samples from microorganisms phylogenetically related to *Leishmania* and those associated with the differential diagnosis of cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL) (Table S1).

To assess the diagnostic performance of the assay in multiple biological matrices, DNA obtained from skin scrapings (extracted with the High Pure PCR Template Preparation kit) and paraffin-embedded tissue (extracted with the QIAamp DNA FFPE tissue kit) of CL patients (diagnosed by microscopy and PCR) as well as whole blood (extracted with the High Pure PCR Template Preparation kit) samples of VL patients (Diagnosed by serology and PCR). A total of 70 positive samples, including 10 control samples from non-endemic areas, were analyzed (Table S2). The diagnostic performance was evaluated by calculating sensitivity, specificity, positive and negative predictive values, and likelihood ratios. In addition, we tested the inter-run and intra-run reproducibility of the different sample types.

The analytical sensitivity of the qPCR assay was determined by diluting parasites in human DNA from non-endemic areas. The assay demonstrated linearity and a dynamic range for all *Leishmania* species tested, with no statistically significant differences observed between species (Fig. 1). The calibration curves showed good results and high reproducibility across triplicates. Probit analysis determined the limit of detection (LoD) for all species to be 1 parasite equivalent per milliliter. For the analytical specificity evaluation, the qPCR assay was tested against microorganisms related to *Leishmania* and those associated with the differential diagnosis of CL, MCL, and VL. No amplification was observed in any of the samples, indicating excellent specificity of the assay (Table S1).

As for diagnostic performance, 70 positive samples, including control samples from non-endemic areas, were analyzed. The assay demonstrated a wide range of parasitic loads in CL and VL samples, with slightly higher loads observed in CL samples. There were no statistical differences in parasite load between the CL and VL sample types (Table S2; Fig. 2). However, we were unable to quantify the amount of tissue per specimen type. Nonetheless, there are no apparent differences in the resulting Ct values for the RNAseP. We acknowledge this as a limitation of our study and recommend that future studies take this into consideration.

The test exhibited good reproducibility across three different testing days, with no inconclusive results or statistical differences between replicates (Table S3; Table S4). The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV),

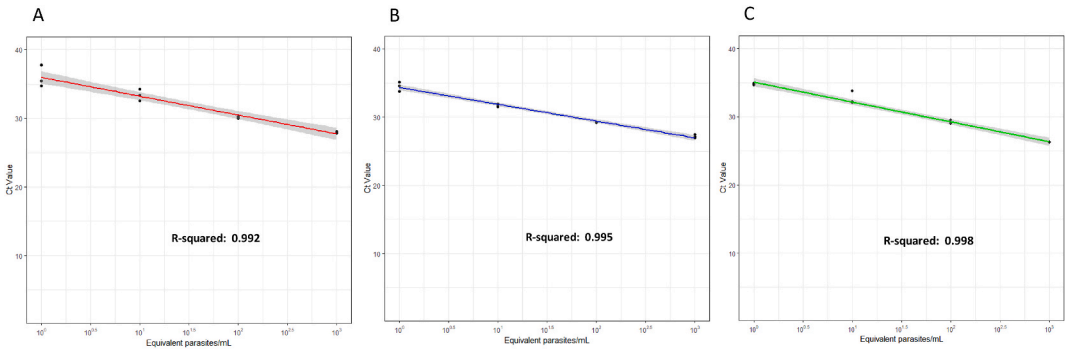

Fig. 1. Dynamic range of the calibration curves using different *Leishmania* species a. *L. braziliensis* b. *L. infantum* c. *L. amazonensis*.

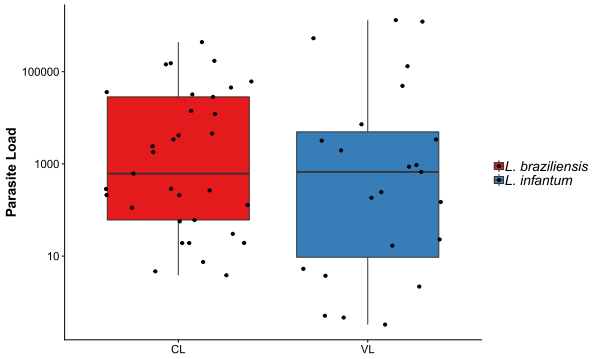

Fig. 2. Parasite load of the CL and VL clinical samples used in the study, each dot represents one sample.

and Kappa index were calculated using reference tests (A compendium of parasitological and molecular tests for CL patients and serological and molecular tests for VL patients). The qPCR assay showed 100 % for all estimated values, indicating excellent diagnostic performance in detecting and quantifying *Leishmania* DNA.

To date, a variety of molecular tools have been developed for accurate diagnosis of cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL). Previous studies have evaluated different molecular markers and methods, but few have comprehensively assessed their analytical and diagnostic performance [6]. One study identified quantitative real-time PCR (qPCR) targeting the kDNA gene as the most sensitive method for *Leishmania* detection, while qPCR targeting ITS-1 and enzyme digestion with *Hae*III plus HSP70 + RLFP were recommended for species identification. Another study compared two commonly used targets (18S rDNA and HSP70) and found high sensitivity and specificity using lesions from CL and mucocutaneous leishmaniasis (MCL) patients [7,8].

In this study, the analytical sensitivity of the qPCR assay was evaluated using three *Leishmania* species, showing a good dynamic range and a limit of detection (LoD) of 1 parasite equivalent per milliliter (eq-p/mL) for all species tested (Fig. 1). Analytical specificity was tested against various microorganisms, demonstrating no amplification and confirming the high specificity of the assay for *Leishmania* DNA (Table S1). For the first time, the assay was used on samples from VL and CL patients, showing complete agreement with the reference methods and enabling estimation of parasite burdens (Fig. 2; Table S2) and highly reproducible (Table S3; Table S4). The results confirmed low parasite loads in VL patients and higher loads in CL patients [9,10]. The study suggests the potential use of this assay for monitoring treatment and following the course of infection in CL and VL patients.

Findings of our study hold practical significance in terms of their implications for laboratory settings by providing a quick and reliable method for pan-genus *Leishmania* detection and pre-screening for the most clinically relevant species in three different sample types (paraffin blocks, skin scrapping and blood). Further studies should include more *Leishmania* species and different clinical manifestations of the disease to improve the clinical validation of the assay including new tools for species identification.

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None.

Ethical statement

The protocol was approved by the London School of Hygiene & Tropical Medicine Observational Ethics Committee (protocol number 15515).

Author statement

JDR and AP conceived the study and drafted the manuscript.
LC, LHP, ACC developed the experiments.
MAS provided samples.
CC and EMS edited the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2023.e00341.

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